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Thermal history parameters drive changes in physiology and cold hardiness of young grapevine plants during winter

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ABSTRACT

Vitis vinifera is mainly cultivated in temperate areas, where seasons are well defined and winter conditions might be severe. To survive under these conditions during the dormant season, grapevines sense environmental parameters to trigger different protective mechanisms that lead to cold hardiness (CH). Crop yield and sustainability will be determined according to the level of CH reached in each organ. Moreover, different cultivars of *V. vinifera* exhibit different behavior throughout the dormant season, attaining a different status of CH. However, there is scarce information concerning how the same cultivar behaves under contrasting thermal environments. The aim of our research was to unveil how CH varies in trunks of the same cultivar under two contrasting environments and define which are the main thermal and biochemical parameters involved in this process. We submitted 2-year old plants of the same clone of cv. Malbec to two different thermal conditions: natural winter (control) and artificially warm winter (treatment). CH status, thermal and biochemical parameters in trunks were measured periodically over the dormant season, and this experiment was repeated for three years. Our results suggest that grapevine trunks subjected to a different environment reach dissimilar CH status, except at the end of winter. In addition, we determined that daily minimum temperature is the main thermal parameter that drives changes in CH. Also, we found that the total soluble sugars have the greatest relative weight in determining the CH compared with the other compounds evaluated. These results have practical implications in the establishment of vineyards for new growing regions. Moreover, with rising minimum temperature predicted by climate change scenarios, grapevines may be more vulnerable to cold events during the dormant season.

1. Introduction

Vitis vinifera is a perennial liana adapted to temperate climates, capable of surviving to relatively low temperatures during the winter. The acquisition of dormancy and cold hardiness (CH) is an active, dynamic and complex process with physiological-biochemical adaptations (Weiser, 1970; Shaulis, 1971; Chen and Li, 1977). Classically, the dormant period is divided into three stages: acclimation, characterized as a period of transition from the non-hardy to the fully hardy state; ii) mid-winter, characterized as a period of most severe cold and greatest CH; and iii) deacclimation, characterized as a period of transition from the fully hardy to the non-hardy state and active growth (Howell, 2000; Ferguson et al., 2014). Traditionally it is thought that during mid-winter CH reaches a threshold of maximum resistance, a factor that is considered constant and independent of weather, even if a warm event occurs (Proebsting et al., 1980; Zabadal et al., 2007; Beck et al., 2004). For that, CH had been described as a U-shaped curve with a maximum hardiness level MHL) thought to be as constant for each year

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(Proebsting et al., 1980). CH is usually measured as the lethal temperature required to kill 50% of tissues (LT_{50}).

Surviving cold temperatures may involve different plant strategies, namely freezing tolerance and/or freezing avoidance, as defined by Levitt (1980). The avoidance of ice formation in plant tissues is linked to cryoprotective compounds that have the function of lowering the freezing point of the cytoplasm (supercooling). Some compounds that were reported to have cryoprotectant properties include simple soluble sugars and free amino acids (Pierquet and Stushnoff, 1980; Guy, 1990; Fennell, 2004). Moreover, tissue dehydration has also been cited as another mechanism to prevent ice formation. The other mechanism is freezing tolerance, which is the capacity to tolerate both ice in the apoplast and the high concentration of solutes in cells (Levitt, 1980).

Grapevine exploits both of these mechanisms. In buds the CH is based predominantly on supercooling but in woody tissues the two mechanisms occur simultaneously (Burke et al., 1976; Andrews et al., 1984; Badulescu and Ernst, 2006). The efficacy of these mechanisms depends on temperature, species, and cultivar in question, meaning that there is a genetic potential of CH (Keller, 2010). It has been reported that *V. vinifera* is not particularly cold hardy, suffering more freeze damage during winter compared with American grapevines species (Londo and Kovaleski, 2017).

Macroclimatic changes may affect local winter conditions. Mendoza Province (MZA), Argentina, between 32° and 36 °South latitude, is the most important grapevine (*V. vinifera*) production region in South America (almost 160,000 ha). Its macroclimate is dry and temperate with a high continentality due to the proximity of the Andes mountain range. This results in a large thermal amplitude over the day/night cycle and between different seasons (Gonzalez Antivilo et al., 2017). Moreover, during winter and spring a strong, dry and warm föhn wind called Zonda is common (Norte and Simonelli, 2016) and may be followed by freezing events that cause injury in fruit trees and reduce plant yields (Caretta et al., 2004).

MZA is a desert region with less than 200 mm/year of precipitation. Therefore, the crops are irrigated with water from melting snow in the Andes, which leads to the crops being concentrated in four small productive oases with different agroclimatic characteristics, partially defined by their geographic location as North, East, Central and South oases (Suppl Fig1A; González et al., 2009; DACC, 2013). More than 20 grapevine cultivars are grown in MZA. The most emblematic cultivar is Malbec which has experienced a strong increase in the last 15 years, more than doubling in the production area to reach 40,000 ha, and distributed in all oases (Instituto Nacional de Vitivicultura, INV, 2016).

According to the IPCC (Stocker et al., 2013) projections, a temperature increase between 2 °C and 4 °C for the next 100 years is expected worldwide. In MZA there has already been an increase in the average minimum winter temperature over the last 50 years (Deis et al., 2015). Other predictions also indicate that climate contingencies will be more extreme, including cold and heat wave events, and a longer frost-free period (Aruani, 2010). Plants will live in a riskier environment if it is fluctuating (Londo and Kovaleski, 2017). In the last decade, hard winters with very low absolute temperatures and late frosts were registered, affecting several production areas in MZA (DACC, 2013). For example, severe freezing events were recorded in large parts of the province, both during the dormant (< -15 °C) and growing (< -4 °C) seasons during 2015 and 2016. By coincidence, the INV indicated grape harvest losses up to 30% for these seasons, compared with the previous harvests. Therefore, as cold injury affects both yield and vineyard sustainability, it is necessary to enhance local information to assist producers and government agencies in zoning existing cultivated areas by variety and in better matching varieties to specific zones at the time of planting a new vineyard.

The objective of this study was to determine if the CH status of *V. vinifera* can be affected by the thermal history during the dormant season. Our strategy consisted in subjecting plants to two contrasting thermal environmental and to evaluate the change in CH in order to establish the relationship between the process of acclimation-deacclimation and different thermal parameters. With this, we tried to establish which parameters explain this relationship better. Moreover, we wanted to unveil periods throughout the dormant season during which the thermal history can influence the maintenance of CH. This information could be linked to agroecological characteristics of each production oases of MZA within the framework of climate change predictions. Finally, we measured the seasonal changes of different physiochemical parameters involved in cold acclimation in order to determine which one may be the most influential in this process.

2. Materials and methods

2.1. Field experiments

2.1.1. Locations and plant material

Three independent experiments were conducted during the winter season (June to September) of the years 2012, 2013, and 2016 (hereinafter referred to as Y-1, Y-2, and Y-3, respectively). During the first two years, assays were carried out in Luján de Cuyo (33° 35′ 24″ S; 68° 30′ 00″ W; 925 m asl), whereas in the third year, it was conducted in Godoy Cruz (32°55′6.69″S; 68°50′32.82″O; 787 m asl), both located in the northern agricultural oases of MZA, Argentina.

For each experiment, 200 2-year-old, own-rooted Malbec certificated clone Perdriel plants were used. Plants were grown in 7-liter pots filled with a mixture of soil:sand:perlite (2:1:1 by vol). During the dormant season (autumn to winter), plants were watered every 15 days. During the growing season (end of winter and spring), at the start of bud burst, watering frequency was increased to twice a week. In order to maintain the canopy in healthy conditions standard pest control strategies were applied until the natural leaf fall. During the months prior to the application of thermal treatments (March to May), plants were grown outdoors.

2.1.2. Thermal treatments, monitoring and ecological characterization

At the beginning of the winter season, plants were randomly divided into 2 groups of 100 plants each. Each group was assigned to a different thermal treatment: natural winter (W_N) and artificially warm winter (W_W). The W_N was considered as control, and consisted of maintaining plants under natural winter field conditions, whereas the W_W treatment consisted of increasing the temperature by using a greenhouse and adding an external source of heat. During Y-1 and Y-2, a 1000 W electric fan heater installed 1 m above soil level was placed within a 2 × 3 m greenhouse coated with 200-micron crystal polyethylene UV protection. In Y-3, a natural gas heater of 3000 cal/hour was placed 10 cm above the ground in a 3 × 4 m greenhouse with the same coating. Heating was performed throughout every night (approximately from 8:00 PM to 7:00 AM of the next day). There were differences in the way heat was applied: i) in Y-1, the electric heater was programmed to be turned on 30 min and turned off 30 min each hour; ii) in Y-2, the heater remained on for 2 h and off for 1 h per cycle; iii) the natural gas heater remained on all night long.

During Y-1 and Y-2, the temperature was monitored using iButton sensors (Thermochron DS1922L-F5 temperature loggers, Maxim Integrated, San Jose, CA, USA, with a measurement range of -40 to + 125 °C, and accuracy ± 0.5 °C); whereas during Y-3, an Arduino mega 2560 logger integrated with DS18B20 sensors developed by IANIGLA-CONICET was used after contrasting and checking with iButton. In all years, two sensors per treatment were installed.

In order to characterize and compare the ecological environments generated by treatments over grapevine physiology during the dormant season (from April 1 until August 31) of Y-1, Y-2 and Y-3, two ecological indices were calculated according to Deis et al (2015): i) ΣTmin, corresponding to the summation of daily minimum temperature (Tmin) during the dormant season and ii) n°D < -3, corresponding to the total number of days that reached temperatures equal to or lower than

−3 °C. These indices were compared with historical records of different production oases in MZA.

2.1.3. Non-destructive and destructive measurements

Phenology was non-destructively assessed on 10 plants randomly selected and labeled specifically for this purpose. Phenological status was determined every 3 days from the beginning of the experiments (June), and classified into three stages according to the modified EL phenological system: dormant (Stage 1, closed or wintering bud), late bud swell (Stage 5, swollen bud or green tip) and bud burst (Stage 7 - bud with expanded or budded first leaf). After bud burst, shoot length was measured at the same frequency for one month.

Destructive sampling was performed every 15 days, approximately, after the start of the experiment. At each sampling date, 8 plants per treatment were randomly selected for laboratory analysis (without replacement).

2.2. Laboratory analysis

2.2.1. Sample processing

In order to avoid sample warming that could lead to deacclimation, sampling collection was done at sunrise. Trunks were cut at soil level, discarding roots and any current-year shoot. Each trunk was placed in an airtight bag with a moist absorbent paper to avoid tissue dehydration. The bags were put in a Styrofoam box until the end of sampling and subsequent transportation to the laboratory.

In the laboratory, trunks were divided into three parts of 5–10 cm each, and used for a different analysis: i) the upper part (Section 1), was immediately frozen in liquid nitrogen and kept at -20 °C until it was used to measure total free amino acids (TFA, measured only in Y-3;) ii) the middle portion (Section 2), was used to measure water content (WC), total soluble sugars content (TSS) and starch content and iii) the basal portion (Section 3) was used to measure CH status; Fig. 1 shows a schematic diagram of the sampling process.

2.2.2. Controlled freezing simulation and CH status determination

For freezing simulations, a commercial freezer Gafa model Eternity S120, controlled by a computer based on Arduino mega 2560 logger equipment was used. The temperature was allowed to decrease from ambient to 10 °C. From this point, the temperature was lowered at -2 °C/h until reaching -19 °C. The temperature was monitored and recorded in real time with an integrated sensor DS18B20.

To measure CH, the basal portion of each trunk was divided into 4 to 5 sub portions of ~3 cm each (Suppl Fig2a-b). All the fragments obtained from the same treatment were mixed, to make a composite sample (SupplFig2c). Then, 10 packages with 5 pieces of trunk each were assembled per treatment and wrapped in aluminum foil. Each package was assigned to a final freezing temperature: Control (not freezing), -2, -4, -6, -8, -10, -12, -14, -16, −18 °C. The packages were removed as simulated freezing progressed (SupplFig2d-e). Upon removal the samples were placed in darkness at 7 °C for 24 h then at room temperature (~20 °C) for another 24 h (SupplFig2h).

The death of phloem and xylem tissues was visually assessed by the browning method, which has been shown to be highly correlated with lethal temperatures determined by differential thermal analysis (Mills et al., 2006). This method is currently a standard procedure to determine CH status in trunks (Aslamarz et al., 2010; Moran et al., 2011). Trunk fragments were transversally cut in halves, then phloem and xylem tissues were observed under a stereomicroscope and classified as alive or dead according to their visual appearance (SupplFig2h-i). The method to estimate LT_{50} in both tissues is described below, in "Statistical analysis" section.

2.2.3. Water content

Fresh weight (FW) was determined using wood material from Section 2 of trunks (Fig. 1) with a precision balance with an accuracy of

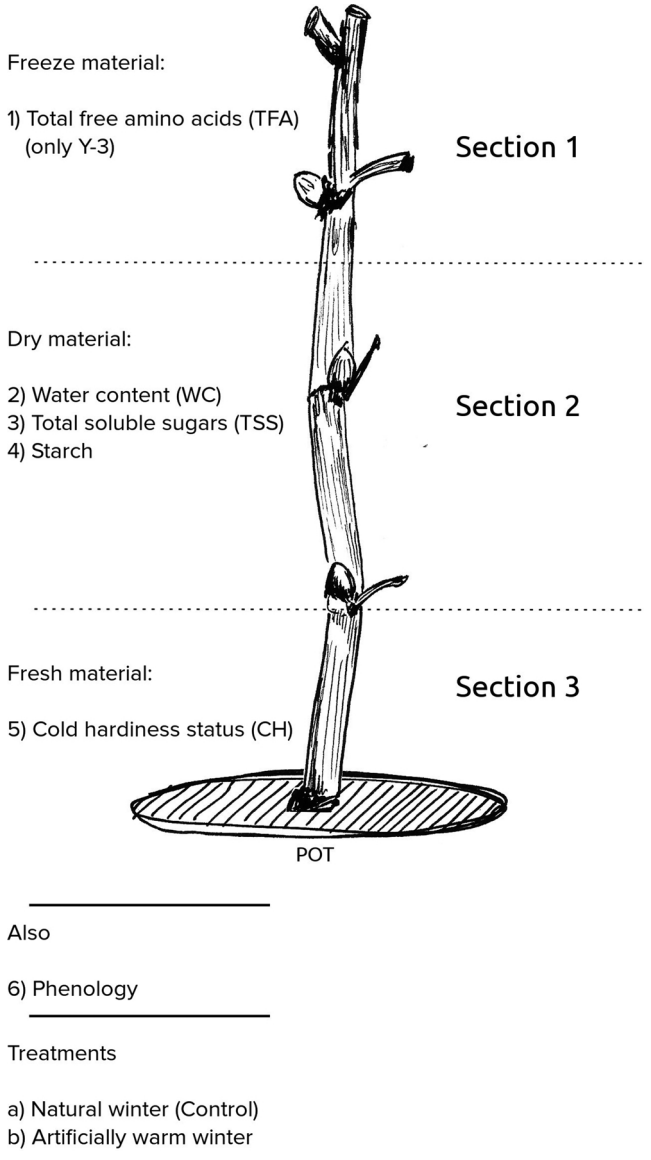

Fig. 1. Trunk sections of two-year-old Vitis vinifera plants. Plants maintained under natural winter conditions (W_N) or artificially warm winter (W_W) conditions were sectioned into three parts for different analyses: i) Section 1, determination of total free amino acids (TFA); ii) Section 2, determination of total soluble sugar (TSS), starch and water (WC) contents; iii) Section 3, evaluation of cold hardiness (CH) status.

0.1 mg. Then, sections were oven-dried at 60 °C for 48 h. Samples were weighed again to determine dry weight (DW). Water content was calculated as follows $WC = (FW\text{-}DW) \times 100 \times FW^{-1}$.

2.2.4. Total soluble sugars and starch content

After determining WC, dry trunk portions of Section 2 were ground with a bladed mill Retsch Z200 (Haan, Germany) to a particle size of < 0.08 mm. Total soluble sugars (TSS) were extracted with 80% ethanol during 45 min. The supernatant was centrifuged at 10,000 rpm for 5 min. Sugars were quantified using the anthrone method (Yemm and Willis, 1954). The pellet was resuspended, digested with perchloric acid, and starch was quantified by spectrophotometry as described by McCready et al. (1950).

2.2.5. Total free amino acids

Total free amino acids (TFA) were determined from Section 1 of trunk as described above. Trunk portions were powdered in a mortar

with the addition of liquid nitrogen to avoid thawing of the samples. TFA content was determined spectrophotometrically by the ninhydrin method described by Yemm and Cocking (1955).

2.3. Statistical analysis

For comparison of thermal parameters among years and treatments, mean values of mean temperature (Tmean), minimum temperature (Tmin), maximum temperature (Tmax) and thermal amplitude (TA) were calculated using daily records during the winter season of years Y-1, Y-2 and Y-3 (n = 90 days per year). For the analysis of differences between thermal parameters, one way-ANOVA was used. When effects were significant, multiple comparisons were performed using DGC method with $\alpha = 0.05$ (Di Rienzo et al., 2002).

For the LT_{50} analysis in phloem and xylem ($LT_{50\text{-}P}$ and $LT_{50\text{-}X}$, respectively) each trunk was recorded as ´alive´ or ´dead´ (binary family). Then, the proportion of dead tissues (P_{Pdead} and P_{Xdead} for phloem and xylem respectively) was calculated for each freezing dose temperature and winter thermal treatment (n = 5; SupplFig2h-i). Adjusted curves of damage proportion were obtained using a logistic regression by generalized linear mixed model (GLMM; co variable: freezing temperature dose; fixed effects: temperature treatment) in each year. The statistical comparison was made between curves, and effects were considered significant if $p < 0.05$. The $LT_{50\text{-}P}$ and $LT_{50\text{-}X}$ were obtained using the equation $\pi = (\exp(\eta))/(1+\exp(\eta))$, where η is the proportion of dead tissue. The relationship between thermal history and $LT_{50\text{-}P}$ and $LT_{50\text{-}X}$ was tested using Pearson's correlation analysis. For this, the mean values of Tmax, Tmin, Tmean and TA were used considering the records of 1, 3, 5, 10 and 15 days prior to the simulated freezing event. In the same way, a Pearson correlation between CH and WC was also performed. In this case, data were partitioned into two parts, one of them including only those data that showed statistically significant differences between treatments in CH, and the other one including only those data without differences among treatments in CH. In both cases, correlations were considered significant at $p < 0.05$. For biochemical parameters, curves of changes in TSS, starch and TFA were made using mean values and standard deviation (n = 8). In order to compare the effect of treatment over these curves, a generalized linear model (GLM; co variable: time; fixed effects: treatment, sampling and interaction) was used. This model estimates statistical differences between curves ($p < 0.05$). The analyses were partitioned by year.

Principal Component Analysis was performed, including three years of measurements of thermal (Tmax, Tmin), physiological ($LT_{50\text{-}P}$ and $LT_{50\text{-}X}$) and biochemical (WC, TSS, starch) variables potentially associated with CH. Also, a MANOVA was done, and when significant, a mean comparison was conducted by the Hotelling test, and differences were considered significant if $p < 0.05$. All the analyses were done using Infostat v 2017 (Di Rienzo et al., 2017).

3. Results

3.1. Thermal characteristics of natural and artificial warming treatments

Two contrasting environments were generated with the W_N and W_W treatments during each winter season of the three analyzed years (Table1). Differences were significant in almost all the thermal variables and years analyzed. The differences between thermal treatments ranged from 3.3 to 5.1 °C for Tmean, 13.0–13.4 °C for Tmax, 0.6–2.6 °C for Tmin, and 10.5–12.7 °C during the three years. Inter-annual differences among W_N were also observed, mainly with respect to Tmin variation. Whereas Y-2 was the coldest year, Y-3 was the warmest and Y-1 showed an intermediate behavior (Fig. 2, Table1, SupplFig3a-b). In Y-1 there was no significant difference in the average Tmin between treatments, in Y-2 the difference was 1.9 °C and in Y-3 it was 2.6 °C. On the other hand, the difference of average Tmax between W_N and W_W was constant in the three years (13–14 °C).

Table 1
Thermal parameters comparison during the winter season between two contrasting thermal environments: natural winter (W_N) and artificially warm winter (W_W). Mean values of mean temperature (Tmean; A), minimum temperature (Tmin; B), maximum temperature (Tmax; C) and thermal amplitude (TA; D) were calculated using daily records during the winter season of the years 2012 (Y-1), 2013 (Y-2) and 2016 (Y-3). ns indicate non-significant differences. Different letters within columns indicate statistical differences between the years.

A)	Tmean (°C)		
	Treatment		
Year	W_N	W_W	p
Y-1	9.1 ± 0.4 [b]	12.4 ± 0.4 [b]	< 0.0001
Y-2	7.4 ± 0.4 [c]	11.9 ± 0.4 [b]	< 0.0001
Y-3	11.2 ± 0.4 [a]	16.3 ± 0.4 [a]	< 0.0001
B)	Tmin (°C)		
	Treatment		
Year	W_N	W_W	p
Y-1	1.8 ± 0.3 [b]	2.4 ± 0.3 [b]	*ns*
Y-2	0.3 ± 0.3 [c]	2.2 ± 0.3 [b]	< 0.0001
Y-3	6.1 ± 0.3 [a]	8.7 ± 0.3 [a]	< 0.0001
C)	Tmax (°C)		
	Treatment		
Year	W_N	W_W	p
Y-1	20.0 ± 0.6 [a]	33.4 ± 0.8 [a]	< 0.0001
Y-2	17.0 ± 0.6 [b]	30.0 ± 0.8 [b]	< 0.0001
Y-3	16.7 ± 0.6 [b]	31.0 ± 0.8 [b]	< 0.0001
D)	TA (°C)		
	Treatment		
Year	W_N	W_W	p
	18.3 ± 0.5 [a]	31.0 ± 0.8 [a]	< 0.0001
	17.4 ± 0.5 [a]	27.9 ± 0.8 [b]	< 0.0001
	10.5 ± 0.5 [b]	22.3 ± 0.8 [c]	< 0.0001

3.2. Ecological indices

There were differences in ΣTmin between W_N and W_W in all years. The modified thermal environment (W_W) increased values of this index (SupplFig1c) by 20, 130 and 33% for Y-1, Y-2 and Y-3 respectively. Under W_N, ΣTmin was much higher in Y-1 (422 °C) than in Y-2 (214 °C). Conversely, W_W recorded very similar index values (ΣTmin: 516 °C and 499 °C in Y-1 and Y-2 respectively). In Y-3, in which the experiment was conducted in a warmer location, ΣTmin was much higher in both treatments (W_N = 912 °C and W_W = 1214 °C). No differences were observed in n°D < -3 between thermal treatments in Y-1 and Y-3. However, the interannual variation was very marked between these two years (n°D < -3 = 15 and 0 respectively). Conversely, Y-2 was the most contrasting: there were nearly 3 times more days with temperatures below −3 °C in W_N than in W_W.

3.3. Phenology

In the 3 years of measurement vines in the W_W treatment were the first to burst each spring, but the timing varied among years. In the warmer year (Y-3) the difference in bud burst time between thermal treatments was more than 30 days, whereas it was only 17 days in the coldest year (Y-2). In Y-1, plants showed an intermediate behavior (Table2).

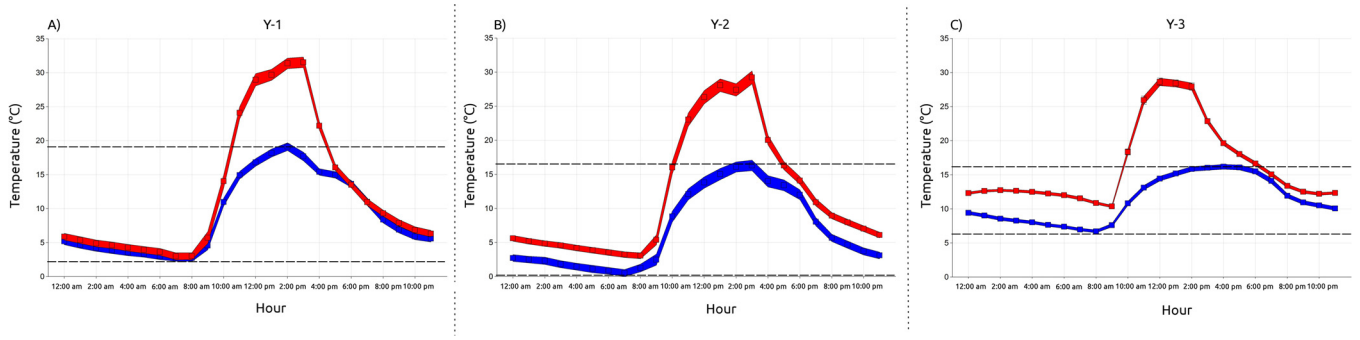

Fig. 2. Daily thermal environment comparison between natural winter conditions (W_N; blue line) and artificially warm winter (W_W; red line). Tmax and Tmin were registered hourly during the 3-month winter season in 2012 (Y-1; A), 2013 (Y-2; B) and 2016 (Y-3; C). The thickness of the line denotes temperature variability between winter days (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 2
Beginning of bud burst (Stage 7 according EL system) in *Vitis vinifera* subjecting under two contrasting thermal environments during three winters, 2012, 2013 and 2016 (Y-1, Y-2, Y-3 respectively): natural winter conditions (W_N) and artificially warm winter (W_W).

	Stage 7: bud burst		
Year	W_N	W_W	Diff (days)
Y-1	Sept-17	Aug-24	24
Y-2	Sept-12	Aug-26	17
Y-3	Aug- 12	Jul-11	32

3.4. Winter CH of trunk phloem and xylem from plants with different thermal history

3.4.1. CH status

A similar pattern was observed across years in CH of trunk phloem (measured as $LT_{50\text{-}P}$) throughout the winter, with significant differences among sampling dates and treatments (Table3, SupplFig3c, SupplFig4). During early- and mid- winter significant differences were observed in phloem CH between W_N and W_W ($LT_{50\text{-}P}$ ranges in all years were -16.5 to -12.1 °C, and -14.5 to -8.2 °C for W_N and W_W treatments, respectively). Plants grown under warm conditions were less resistant to cold than those grown under natural conditions (Table 3a). This difference disappeared about one month before the beginning of spring. In addition, the climatic conditions of each year also appeared to affect phloem CH. Thus, Y-2 (the coldest year) was associated with lowest $LT_{50\text{-}P}$, whereas in Y-3 (the warmest year) the highest $LT_{50\text{-}P}$ values were reached (Table 3a).

Results of LT_{50} in xylem are less clear than those observed in phloem (Table 3b, SupplFig3; SupplFig5). In several cases, cold hardiness exceeded the range of temperatures tested in the freezing simulation (Table 3b). In any case, xylem was more cold hardy than phloem (range of $LT_{50\text{-}X}$: -13,3 down to < -19.0 °C). Moreover, the difference between treatments disappeared later in xylem than in phloem, lasting up to the beginning of spring (Table 3b).

3.4.2. Thermal parameters and their correlations with CH

In order to explore how the thermal history influences the cold resistance of the trunk correlations between CH ($LT_{50\text{-}P}$ and $LT_{50\text{-}X}$) and the averages of Tmin, Tmax, TA and Tmean of 5 periods prior to the freezing simulation (1 day, 3 days, 5 days, 10 days and 15 days) were calculated (Fig. 3). These correlations were significant in both tissues and always positive, indicating that lower temperatures were associated with hardier trunk tissues, independent of the number of days used for the analysis. However, the correlation coefficient increased as more days were considered in the analysis in both tissues. The variation in Tmin explained more of the variation in CH status than did that in Tmax. Other variables such as TA and Tmean were not correlated with CH status under our conditions (data not shown).

Table 3
Three-years measurement of lethal temperature (LT_{50}) induced by thermal treatment (W_N and W_W) on phloem ($LT_{50\text{-}P}$) and xylem ($LT_{50\text{-}X}$) of *Vitis vinifera* during the winter season (2012 as Y-1, 2013 as Y-2 and 2016 as Y-3).

A

LT50 Phloem (°C)					
Year	Date	p	N_W	W_W	Diff
1	August-2	*	−12.4	−10.8	1.6
	August-18	*	−14.4	−11.9	2.5
	September-11	*ns*	−8.5	−8.0	0.5
2	June-25	*	−14.0	−12.0	2.0
	July-16	*	−16.4	−14.5	1.9
	August-10	*	−16.5	−11.2	5.2
	August-31	*ns*	−13.1	−12.0	1.1
	September-18	*ns*	−12.3	−11.9	0.4
3	June-24	*	−16.3	−9.2	7.0
	July-18	*	−12.1	−8.2	3.9
	August-8	*	−12.3	−9.2	3.1
	August-28	*ns*	−9.3	−7.0	2.3
	September-13	*ns*	−10.5	−10.5	0.0

B

LT50 Xylem (°C)					
Year	Date	p	N_W	W_W	Diff
1	August-2	*ns*	−18.8	−17.5	1.3
	August-18	*	−19.6	−15.6	4.0
	September-11	*ns*	−13.8	−12.8	1.0
2	June-25	*ns*	−18.3	−16.1	2.2
	July-16	*nd*	< -19.0	−16.6	< 2.4
	August-10	*	−18.8	−16.1	2.7
	August-31	*	−18.8	−15.5	3.3
	September-18	*ns*	−15.2	−13.6	1.6
3	June-24	*nd*	< -19.0	−15.0	< 4.0
	July-18	*	−17.5	−11.5	6.0
	August-8	*	−17.7	−13.9	3.8
	August-28	*	−17.2	−12.6	4.5
	September-13	*ns*	−13.3	−12.5	0.8

nd: non determined; *ns*: non significant; *: significant differences (for significance values, see SupplFig4 and SupplFig5); Diff means the difference between LT_{50} artificially warm winter (W_w) and LT_{50} natural winter (W_N).

3.5. Physiological and biochemical variables

Plants in W_N always had higher TSS than in W_W (Fig. 4), despite large variation in W_N and W_W conditions among years. Sugars decreased when plants entered into the bud burst phase. Moreover, the effect of thermal treatment on sugar content became not significant when plants began to deacclimate in late winter and start of spring,

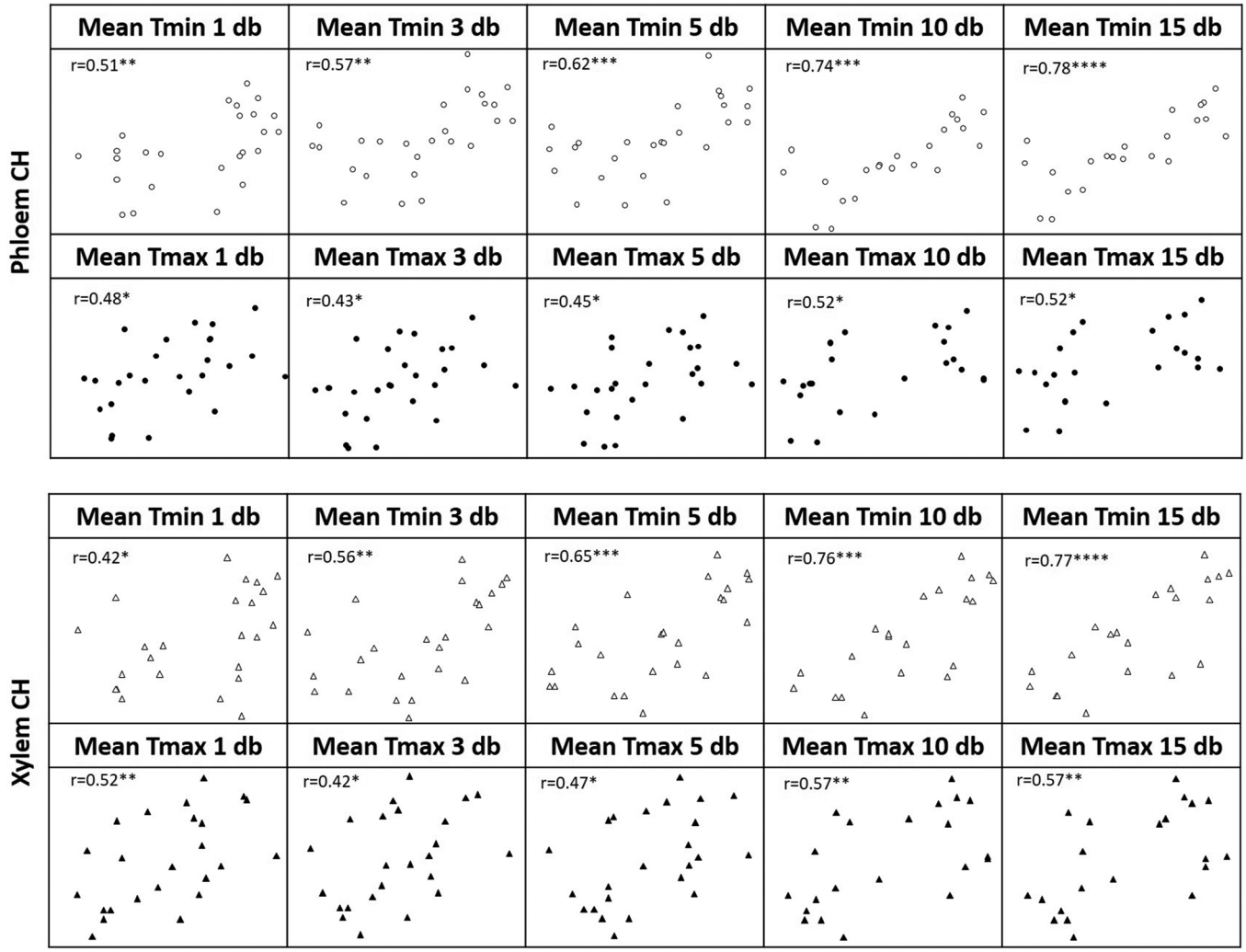

Fig. 3. Correlation analysis of the mean Tmax and Tmin registered at 1, 3, 5, 10 and 15 days prior to the measurement of the cold hardiness status in trunk phloem (A) and xylem tissues (B). Analysis was performed over three years during the winter season (n = 3 years; 2012, 2013 and 2016). The number of asterisks indicates the significance level of correlation analysis: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$ and **** $p < 0.00001$.

approximately from September 22 (Fig. 4).

In Y-1 and Y-2, bud burst occurred at a stage in which CH was not significantly different between treatments (Fig. 4a-b). The Y-3 was the only one that presented significant differences in sugars even though the buds had already begun bursting (Fig. 4c).

In contrast with TSS, there was no correlation between starch and CH status (data not shown). The starch concentration varied between 6 and 13% during the analyzed period. The effects of thermal treatments on trunk starch were inconsistent and generally not significant.

Total free amino acid content (TFA) was only analyzed in Y-3. Under W_N conditions, TFA concentrations remained virtually constant throughout the analyzed period (SupplFig6). On the other hand, in W_W the TFA declined almost five-fold during bud burst. No clear relationship was found between amino acids, CH and the thermal history.

3.6. Principal components analysis (PCA)

In order to determine the importance of each one of the evaluated variables for the CH status of vines exposed to different thermal histories, we performed a PCA (Fig. 5) of the entire dataset. The 1st and 2nd canonical components explained together 67.5% of the total variation. The largest variation was explained by component 1 (51.9%), where the canonical variables of W_N (left quadrant; white symbols) and W_W (right quadrant; black symbols) were mainly separated. The TSS was strongly associated with W_N and exhibited a strong negative correlation with Tmin. The CH in phloem ($LT_{50\text{-}P}$) and xylem ($LT_{50\text{-}X}$) was associated with the thermal history; higher Tmax or Tmin values (thermal history of 10 days) were associated with less negative LT_{50} values. Component 2, on the other hand, accounted for only 15.4% of the variation, and starch varied along this axis, which exhibited low association with CH. Water content was partially related to the W_W treatment, indicating that the higher the WC, the lower the degree of cold acclimation. The MANOVA indicates that thermal treatments were significantly different.

4. Discussion

4.1. Cold hardiness status and its relationship with thermal history

One of the patterns that describe CH status during the dormant season is the typical U-shaped curve (Proebsting et al., 1980). Currently, the real-time monitoring system of CH status carried out by Washington State University confirms that sort of curve in different *V. vinifera* varieties (http://wine.wsu.edu/extension/weather/cold-hardiness/). Most of this research has been performed in sites exposed to a low TA and where sometimes extreme Tmin conditions occur (Proebsting et al., 1980; Mills et al., 2006; Cragin et al., 2017). However, there are other studies that support the notion that CH fluctuates

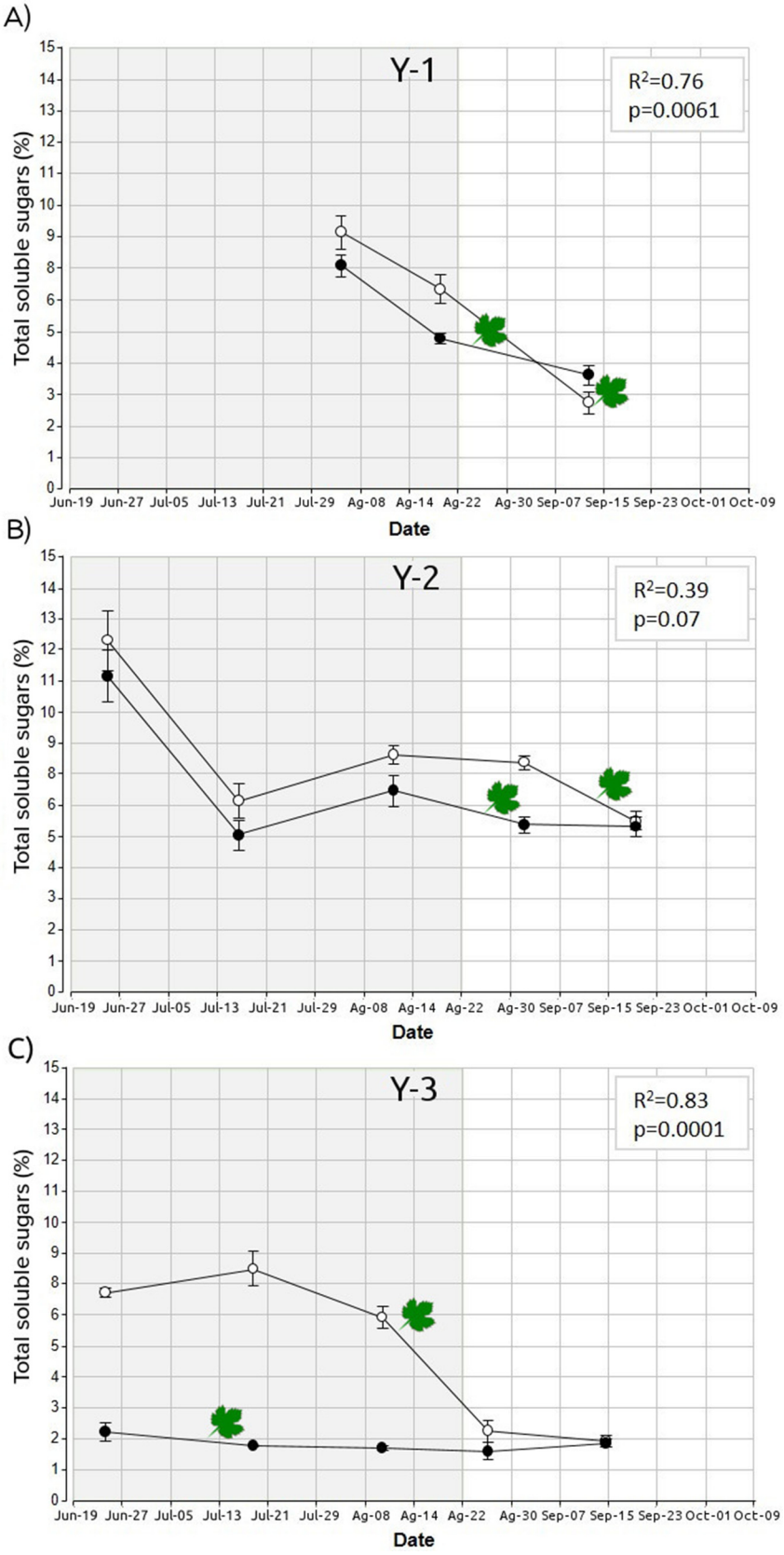

Fig. 4. Three-year changes of percentage of total soluble sugars in trunks of *Vitis vinifera* plants submitted to two different thermal environments during the winter season: natural winter (W_N control, ○) and artificially warm winter (W_W, ●). Measurements were performed every 15 days and repeated during three years: 2012, 2013 and 2016 (Y-1, Y-2 and Y-3 respectively; n = 8 plants per treatment and sampling date). Shaded box indicates the winter period where there is a significant effect of temperature on cold hardiness. Leaves indicate the beginning of bud burst in each treatment.

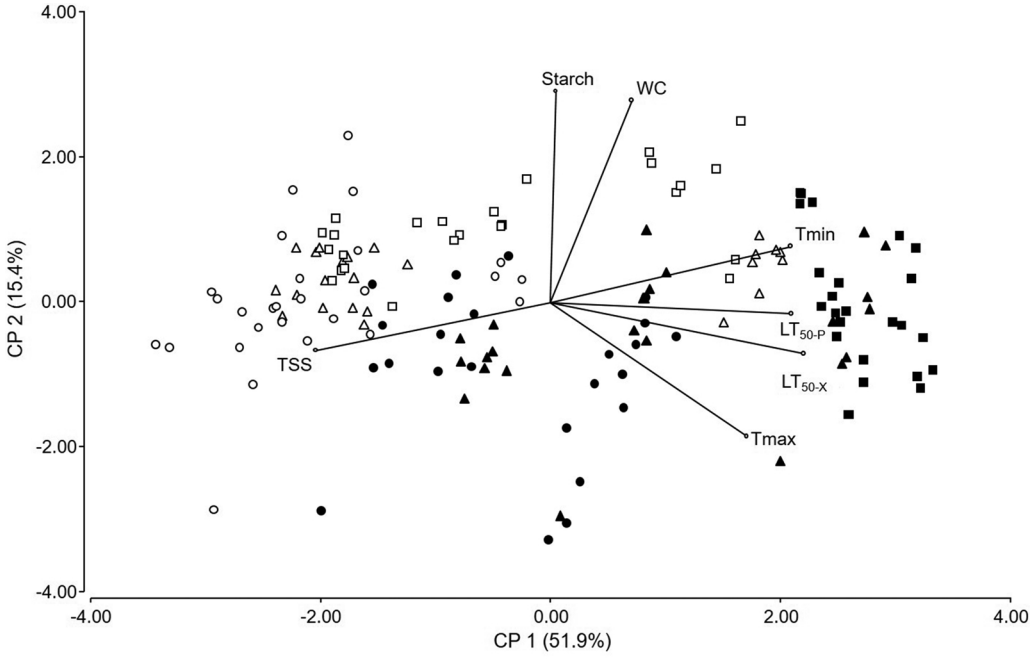

Fig. 5. Principal Component Analysis (PCA) including three years of measurement of thermal (Tmin and Tmax), physiological ($LT_{50\text{-}P}$ and $LT_{50\text{-}X}$) and biochemical variables (starch, total soluble sugars or TSS, and water content or WC) involved in cold hardiness of *V. vinifera*. White symbols correspond to W_N (Y1-△; Y2-□; Y3-○) and black symbols to W_W (Y1-▲; Y2-■; Y3-●) treatments. Table below PCA shows MANOVA results followed by means comparisons by Hotelling test. Different letters indicate differences among treatments ($p < 0.05$).

Significant Roy MANOVA test (p<0.0001). Means comparison by Hotelling´s test.

Treatment	WC	TSS	Starch	PLT50	XLT50	Tmin	Tmax	n	
W_N	47.6	6.7	10	-13.1	-17.7	2.2	17.1	76	A
W_W	47	4.9	9.1	-10.5	-14.8	4.6	31.2	73	B

Means with different letter has significant differences (p<0.05)

depending on the thermal conditions of the season every year (Hubácková, 1996; Vitra et al., 2017). In addition, maximum CH may not be automatically reached in every year, but may rather depend on the thermal history to which grapevine is subjected, which may or may not allow reaching the full genetic potential of each cultivar (Schnabel and Wample, 1978; Fennell and Hoover, 1991; Moran et al., 2011; Londo and Kovaleski, 2017). In the 3 years measured in the present work, there were differences in CH that could be explained as a function of the thermal history, where the coldest year recorded the greatest resistance and the warmest year the least (Table 3; SupplFig3).

We subjected young plants of the same clone of *V. vinifera* to two different thermal environments, controlling the remaining conditions. Our results showed that the thermal history affects CH during the early- and mid-winter stages, where the colder environment (W_N) leads to higher CH status (lower LT) of trunk vascular tissues. However, this difference was lost about one month before the beginning of spring in trunk phloem, while CH in xylem was maintained for a longer period until the end of winter (Table 3; SupplFigs. 3–5). Therefore, this late period of the dormant season is, independently of temperatures, the most susceptible to cold events in vineyards. In the particular case of MZA, this stage requires particular attention because it is precisely the period of the year that experiences the highest incidence of Zonda winds that generate conditions of wide thermal amplitude and are generally followed by strong frosts (Caretta et al., 2004) which can produce damage in trunk vascular tissues (Gonzalez Antivilo, 2018).

Despite past efforts to understand the underlying mechanisms of CH changes through the dormant season, they are still not clear at all, and there is no certainty around what is the main thermal parameter driving this process. Several studies in *V. vinifera* imply that Tmean of the previous day is the main factor involved (Ferguson et al., 2011, 2014); however, other studies did not find such a relationship (Badulescu and Ernst, 2006). Hubácková (1996) found a high correlation with Tmean but considering a thermal history of 15 days. Their results suggest that Tmax explains better the changes in CH, whereas no correlation with Tmin was found. Londo and Kovaleski (2017) found that the best correlation with CH was given using a 7-day thermal history by employing a temperature index calculated using hourly temperatures. In our case, when correlating our three years of measurement of CH (in both xylem and phloem) with different thermal parameters, we found that Tmax and Tmin were the ones that best explained the changes in CH when only a single day was considered prior to our simulated freezing event (Fig. 3). However, when more days were considered, the correlation of Tmin with CH increased considerably (phloem: $r_{1day} = 0.51$; $r_{15day} = 0.78$; xylem: $r_{1day} = 0.42$; $r_{15day} = 0.77$) whereas when evaluating Tmax, the increase of days did not improve the explanation of CH variation. Similar results were found in walnut (Poirier et al., 2010).

4.2. Physicochemical changes associated with to cold acclimation and deacclimation processes

Many quantitative and qualitative physiochemical modifications can occur in response to cold. Most plant species accumulate different kinds of compatible solutes such as soluble sugars and amino acids under stress (Guy, 1990; Close, 1997). We attempted to analyze the weight of these components relative to CH status. We found that TSS are strongly related with CH, whereas WC exhibited a weaker relationship. We did not identify any relationship between CH and either TFA or starch content.

Soluble sugars constitute some of the most well-known compatible solutes during freezing events, since they are involved in the supercooling process (Chen and Li, 1977; Hamman et al., 1996; Howell, 2000; Badulescu and Ernst, 2006). In *V. vinifera*, soluble sugars content has been observed to change in concordance with acclimation to low temperatures in buds and canes (Hinesley et al., 1992). Our results suggest that similar responses occur in young grapevine trunks. This means that TSS accumulation is a dynamic and variable process that

occurs in different tissues and organs of grapevine. Keller (2010) suggested that the soluble sugars reach a peak in accordance with the coldest temperatures during mid-winter. Contrary to the prevailing opinion, TSS were unrelated to CH in some woody plant species, i.e., high levels of total sugars did not necessarily coincide with increases in CH (Stushnoff et al., 1993). Beck et al. (2004) mentioned that in forest species the sugar content between an acclimated plant and a deacclimated plant can be up to 4-folds different. In our research, we found a close relationship between the TSS content and CH status during the first two months of winter. In concordance with Beck et al. (2004) the differences found here were close to 4-fold but only in the most contrasting year (Y-3, Fig. 4). However, these differences became weaker through the last part of the dormant season (Fig. 4). This suggests that sugar content varies according to yearly climatic characteristics but also in different stages of the dormant season in the same year.

The tissue WC is also considered a factor that is highly correlated with resistance to cold (Howell, 2000; Poirier et al., 2010). We found some correlation between CH status and WC (Fig. 5). Several species reduce the water content of their tissues during the dormant season to values similar to our results in order to reduce freezable water (Chen and Li, 1977). This has also been observed in *V. vinifera* (Parostarchy et al., 1980) and other fruit species (Kang et al., 1997). In fact, Chen and Li (1977) reported that by forcing dehydration for a period of 21 days, tissues can quadruple their resistance to cold.

Regarding amino acids, several studies suggest that they act as compatible solutes in a similar way as soluble sugars, participating in the supercooling process. Among amino acids, proline has been frequently found to increase during stress, and has been described as cryoprotectant in many species (Duncan and Widholm, 1987; Guy, 1990; Ait Barka and Audran, 1997). However, in the present work, although a modulation of the amino acid content was observed throughout the winter due to temperature fluctuations, no clear relationship with CH could be established.

4.3. Agroecological regions in the context of climate change

Most agroclimatic indices are constructed on basis of the growing season period. Much less emphasis has been given on estimating indices for the dormant season (for a recent example see Badr et al., 2018). Such indices could be used to measure the sustainability of a variety under inclement weather during winter and at the time of bud burst (Mosedale et al., 2015). Deis et al. (2015) published several ecological indices for the winter using 10 years of thermal records in MZA (2001–2010) and demonstrating large differences among the different oases (SupplFig. 1c). Moreover, our results show that there are marked differences among years for the same site.

One of the aims of this research was to artificially alter natural agroclimatic conditions using heating systems to emulate warmer agroecological scenarios (Fig. 2, Table 1, SupplFig. 1) as is expected for MZA (Deis et al., 2015). In this way, our artificial system (W_W) was capable to increase ΣTmin compared with natural conditions (W_N) for each year (20, 130 and 30% increase in Y-1, Y-2 and Y-3 respectively). It should be noted that in Y-3, our ΣTmin results were higher in W_N and W_W than those reported historically in the warmest region of MZA (East oases; SupplFig2 C; Deis et al., 2015).

An increase in late-winter temperature can advance the time of bud burst, increasing the risk of spring frost damage (Mosedale et al., 2015). Variations in the average date of bud burst have been reported among the different productive oases of MZA, first bud burst the North oases (Sept-22), followed by the East oases (Sept-25) and later in the Central oases (Oct-6) (DACC, 2013). In each year in this work, the W_W treatment induced an early bud burst with respect to W_N. The difference was 17 days in the coldest year (Y-2) and 32 days in the warmest year (Y-3) (Table 2).

In the context of climate change, weather alterations are expected to increase the Tmin, decrease the number of cold events and increase the variation of temperatures during winter and the occurrence of unseasonable and extreme warm and cold events (Solomon et al., 2007; Gu et al., 2008; Stocker et al., 2013; Londo and Kovaleski, 2017). These changes might have consequences in crops, such as an extension of the growing season by a delay in leaf fall (Hänninen, 1991; Saxe et al., 2001). Also, this may accelerate bud burst, leaving new shoots more susceptible to late frost (Mosedale et al., 2015). In contrast, frost reduction does not necessarily reduce the risk of frost damage. Instead, it could remain the same or even become greater (Cannell and Smith, 1986; Meehl, 2000; Londo and Kovaleski, 2017). For deciduous forests it was demonstrated that alternating warm and cold waves in spring are more harmful to plants than a consistently cold spring (Gu et al., 2008). Our study of W_W might demonstrate how the same variety reaches a lesser acclimation level and higher susceptibility to freeze damage (Table 3).

It is common for the same grape cultivar to be grown in different regions, and this situation could lead to different CH status even over a distance of a few kilometers. In MZA, as in several other regions around the world, the frontier of grape production has been extended to new regions without a refined knowledge of the influence of the agrometeorological characteristics on CH of each cultivar.

5. Conclusions

Our results obtained from 3-years of assays in plants of *V. vinifera* subjected to contrasting warm winter environments revealed that temperature drives differences in CH status in plants. Both thermal parameters showed a significant positive correlation with CH; however, a 15-days thermal history of Tmin was found to be the main driver of acclimation status. Yet this was true only in the early- and mid-winter, whereas in the last part of the winter, no treatment effect was registered at least in the trunk phloem. On other hand, when the association of various compounds related to CH was analyzed, we found that sugars have the greatest weight, followed by water content. The artificially created environments in this work could perfectly fit to predicted scenarios of climate change, and in this context grapevines would be more exposed to cold damage than at present.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.agrformet.2018.07.017.

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